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Soil temperature and moisture effects on the persistence of synthetic androgen 17α-trenbolone, 17β-trenbolone and trendione

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ABSTRACT

Trenbolone acetate (TBA) is a synthetic androgenic steroid hormone administered as a subcutaneous implant for growth promotion in beef cattle. The primary metabolite excreted in manure from implanted cattle is 17α-trenbolone with lesser amounts of 17β-trenbolone and trendione also present. At 22 °C and favorable moisture conditions in a controlled laboratory environment, trenbolone degrades to trendione in a few hours; however, these conditions are often not what exist in the field. Therefore, aerobic degradation rates of 17α-trenbolone, 17β-trenbolone and trendione were determined in a sandy soil and silty clay loam under a range of temperature and water availability combinations that may be expected in the field. A first-order exponential decay model was used to estimate rates and generally resulted in good model fits to the data. Degradation rates decreased with decreasing water availability (*i.e.*, more negative soil matric potential) and decreasing temperature. However, when water availability was substantially reduced (−1.0 MPa), hotter temperatures (35 °C) significantly reduced trenbolone degradation rates. Once temperature was low enough to limit microbial activity, no further changes were observed with decreasing matric potential. Trendione also exhibited similar moisture and temperature dependent degradation, but persisted longer than the parent trenbolone. The latter was discussed in light of extracellular versus intracellular enzymatic degradation and sorption. Half lives at colder temperatures (5 °C) even under favorable moisture conditions were 2–3 d for the trenbolone isomers and approached 10 d for trendione.

1. Introduction

Steroid hormones are now frequently detected in the environment and have emerged as contaminants of increasing concern (Shore et al., 1995; Nichols et al. 1997; Moore et al., 2000; Kolpin et al., 2002; Ying et al., 2002; Shore and Shemesh, 2003; Shore et al., 2004; Soto et al., 2004; Durhan et al., 2006). Manure produced in intensive livestock operations is one significant source of hormonal loads to the environment (reviewed in Lintelmann et al., 2003). In addition to naturally produced hormones in the range of milligram quantities per animal per day, many farm animals are also treated with synthetic hormones for various purposes. In particular, 17β-trenbolone acetate (17β-acetohydroxyestra-4,9,11-trien-3-one, TBA) is a synthetic androgen used widely as a growth promoter (Montgomery et al., 2001). TBA has been shown to be more potent than the natural androgen testosterone in terms of binding affinity to the human androgen receptor (Bauer et al., 2000). The expansion and intensification of large-scale concentrated animal feeding operations (CAFOs) in the United States has led to an increased use of anabolic steroids such as TBA (Montgomery et al., 2001; Lone, 1997). Increased use of TBA and detection of TBA metabolites in water bodies (Soto et al., 2004; Durhan et al., 2006) have led to an increased interest in the environmental fate and possible effects of TBA associated compounds on aquatic wildlife. Aquatic organisms exposed to trenbolone have demonstrated biological responses such as reduction in plasma vitellogenin concentration, development of secondary sexual characters, reduced fecundity and masculinization of female fish (reviewed in Kolok et al., 2008; Sellin et al., 2009; Yohana et al., 2010).

TBA is hydrolyzed in the bloodstream of cattle to active 17β-trenbolone (17β-hydroxyestra-4,9,11-trien-3-one) followed by oxidation to trendione (17β-hydroxyestra-4,9,11-trien-3,17-one) and reduction to 17α-trenbolone (17α-hydroxyestra-4,9,11-trien-3-one) (Pottier et al., 1981). Manure excreted from TBA-implanted cattle contains all three metabolites, but 17α-trenbolone is by far the major metabolite. Schiffer et al. (2001), e.g., reported levels in beef dung up to 75 μg kg^{-1} of 17α-trenbolone whereas 17β-trenbolone and trendione were not more than 5 μg kg^{-1}.

The fate of trenbolone and its metabolites in soil after manure is land applied will directly control the level of these hormones available to impact wildlife. Upon mixing manure with soil, hormones undergo additional processes including release from the manure,

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Table 1
Selected soil properties.

Soil (ID)	pH in water 1:2 g mL	Organic carbon (%)	Clay (%)	Sand (%)	Silt (%)	CEC ($cmol_c\ kg^{-1}$)
Coloma-39 (C39)	5.9	0.62	8	81	11	4.3
Drummer-36 (C36)	7.4	2.3	36	17	47	15.5

binding to soil particles and degradation. Both aerobic degradation and sorption can reduce their persistence and mobility in the environment. Microbial transformation processes play a dominant role on the persistence of androgens and estrogens in soil (Jacobsen et al., 2005; Khan et al., 2008). Microbial activity in soil is strongly dependent on the soil moisture and temperature. In laboratory studies, conducted under temperature and moisture conditions conducive to active microbial communities, the half lives ($t_{1/2}$) of 17α-and 17β-trenbolone at applied concentrations of 0.05 to 1 mg kg^{-1} were relatively short (⩽0.5 d) (Khan et al., 2008). However, hormones in manure amended soil could persist longer under extreme conditions of temperature and limited water availability. For example, in aerobic degradation studies conducted by Lorenzen et al. (2005) in an agricultural soil, testosterone was degraded more slowly at 4 °C than at 23 °C. Similarly, Colucci et al. (2001) found that 17 to 20% of 17β-estradiol applied at 1–10 mg kg^{-1} to a sandy loam was mineralized to CO_2 in 3 d when incubated at 30 °C and 13–15% moisture content. In comparison, only 0.4% of 17β-estradiol was mineralized in the same sandy loam when air-dried. Colucci et al. (2001) also noted that 17β-estradiol mineralization increased from 3.6% to 14.7% with increasing incubation temperature from 4 °C to 37 °C. These findings indicate that longer hormonal persistence is expected under dry soil conditions such as may occur in surface soils after spring manure-applications before planting as well as during colder months of the year typical of post-harvest manure-application.

To date, no such experiments have been conducted to assess biotransformation patterns of the synthetic androgen trenbolone and its metabolites in manure-amended soil under the range of conditions expected in the field. The research presented here focuses on quantifying the effect of moisture content and temperature on the persistence of trenbolone and its metabolites. Aerobic degradation rates were measured in two contrasting manure-amended soils under a variety of moisture and temperature conditions ranging from microbially active (−0.03 MPa and 25 °C) to more microbially-stressed conditions (−1.0 MPa and 35 °C, or 5 °C). Most of the work focused on the major excreted metabolite 17α-trenbolone with 17β-trenbolone and trendione persistence assessed under a limited set of moisture-temperature combinations.

2. Materials and methods

2.1. Chemicals

17β-Trenbolone and 17α-trenbolone were obtained from Sigma Chemical, St. Louis MO, USA and Hayashi Pure Chemical IND., LTD., Japan and stored at 4 °C. Other chemicals used included acetonitrile, methanol, and dichloromethane, which are all of >99% purity. Trendione was not available commercially, thus was synthesized (see Supporting Material for details).

2.2. Soils and soil microcosms

Two soils with distinctly different textures and % organic carbon (OC) (Table 1) were sampled from two agricultural fields near at the Purdue Animal Science Research and Education Center (ASREC) (West Lafayette, IN) where effluent and manure-applications are periodically applied. Each soil sample represented a mix of multiple subsamples taken from the surface (top 5–8 cm) at least 1–1.5 m apart from the sampling area. Soils were moist sieved (2 mm maximum particle size) and stored moist at 4 °C prior to use.

Aerobic biodegradation studies were conducted in 40 mL glass vessels with Teflon-lined screw caps. Soil (∼ 5 g dry wt.) samples were placed in the microcosms and adjusted to a range of moisture values from air-dried (−1.0 MPa to −0.03 MPa, field capacity), with incubation temperatures of 5 °C, 25 °C and 35 °C (Table 2). Microcosms were amended with manure from beef cattle that had been free of TBA implants for 4 months. Manure was mixed into soil at a rate of 0.1 mL per ∼ 5 g soil, which corresponds to approximately 20 tons $acre^{-1}$. Land application rates of manure are based on crop nitrogen needs and the amount of nitrogen in the manure, and typically range from 4 to 70 tons $acre^{-1}$ for cattle manure (Iverson and Davis, 2000). After manure addition, all samples were pre-incubated at 22 ± 2 °C for 72 h and then for another 24 h at the targeted incubation temperatures (5 °C, 25 °C and 35 °C) prior to hormone addition.

2.3. Hormone addition, sample extraction and concentration

After pre-incubation with manure at the desired temperature, hormone was added to soil by mixing in 50 mg of hormone-coated talc powder, which resulted in a soil hormone concentration of 50 μg kg^{-1}. Talc powder was coated with hormone by weighing 4 g of talc into a Petri dish, mixing in 2 mL of a 10 mg L^{-1} hormone solution in ethanol of 17α-trenbolone, 17β-trenbolone or trendione, and evaporating off the solvent. The amount of talc added was (50 mg) which accounts for only 1% of the total soil weight. In a previous study, we observed no significant difference in trenbolone or trendione degradation between using talc versus ethanol as the carrier for hormone addition to soil (Khan et al., 2008) (also shown in Supporting Material, Fig. S1).

A set of generally triplicate microcosms were sacrificed for extraction at 9–10 designated times over a 9–10 d period. Samples were extracted with methanol (35 mL) on a rotary shaker overnight and centrifuged at 600 g for 30 min. This method yielded an extraction efficiency of 95–100% as previously determined by Khan et al. (2008) (also shown in Supporting Material, Fig. S2). A

Table 2
Summary of incubation temperature and matric potential conditions.

Matric potential (MPa)	Corresponding moisture content (%)		Incubation temperature (°C)
	D36	C39	
−0.03	26	10	5
−0.1	22.3	8	5
			25
−0.5	14	5	25
			35
−1.0	11.8	3	35

2 mL supernatant aliquot was taken and concentrated to 0.5 mL by evaporating the solvent under a gentle stream of nitrogen.

2.4. LCMS analysis

Analysis was done by gradient elution high pressure liquid chromatography (HPLC) using a Shimadzu HPLC coupled with a Sciex API3000 mass spectrometer detector. Multiple reaction monitoring (MRM) mode was used for quantification of the trenbolone isomers (precursor ion 271, product ion 199) and trendione (precursor ion 269, product ion 225). Separation was performed using 15 μL injections on an end-capped Phenonemex Hyperclone ODS column (150 × 2.0 mm, 3 μm particle diameter and pore size of 130 Å) with an acetonitrile/methanol–water gradient elution at a flow rate of 0.25 mL min^{-1}. The gradient elution started with an composition of 65% solvent A [water: methanol (90:10)] and 35% solvent B [acetonitrile] followed by a linear gradient to 70% B from 3 to 6.5 min after which solvent B was ramped to 96% B for 1.5 min to wash the column and then re-equilibrated at 35% B prior to the next injection. The chromatographic retention times were 3.95 min, 4.65 min and 5.5 min for 17β-trenbolone, 17α-trenbolone, and trendione, respectively. Matrix effects that are commonly observed with HPLC/MS are usually accounted for by using isotopically-labeled internal standards. However, deuterated forms of the target analytes (17α-trenbolone, 17β-trenbolone and trendione), the preferred internal standards of choice, were not available. Therefore, potential matrix effects were assessed by comparing MS responses between standard analyte solutions prepared in methanol and standards prepared in soil extract solution. Soil extract solutions were prepared by extracting soil (5 g of C32 or D30 soil) in which no hormone was applied, treating extracts in the same manner as all samples. There was no significant difference in instrument response between the analyte standards with and without the soil extract matrix (see Fig. S3 in the supplemental information); therefore external calibration curves were used to quantify each hormone. The on column limit of detection (LOD) was 0.3 pg for each compound (with a 15 μL injection volume, which translate to a solution concentration of 0.02 μg L^{-1}) and allowed detection of soil concentrations as low a 0.16 μg kg^{-1} (details on the determination of the method detection limits are provided in Supporting Materials).

2.5. Degradation rates

Degradation rates (k_a, h^{-1}) were determined using a first-order exponential decay model (Eq. (1)), which was derived assuming irreversible transformation, negligible impact of sorption on degradation, and insignificant microbial growth:

$$C_t = C_0 e^{-k_a t} \quad (1)$$

where C_0 and C_t are the applied hormone concentrations at $t = 0$ and at time t(h). Eq. (2) was used to estimate degradation rates of the subsequent metabolite (k_b, h^{-1}) assuming metabolite degradation was first order, fixing k_a at the value estimated from Eq. (1), and optimizing for k_b:

$$C_t = [C_0 - (C_0 e^{-k_a t})] e^{-k_b t} \quad (2)$$

Degradation rates were estimated with a nonlinear regression analysis of the exponential decay model (Eqs. (1) and (2)) using the Statistical Analysis System (SAS 9.1). Paired t-tests were done in SAS to assess treatment effects on degradation rates by comparing changes in one variable with other conditions constants: varying temperature at constant moisture content for a given soil and chemical; varying moisture at a constant temperature; contrasting

Table 3
Aerobic degradation rates (k_a and k_b, h^{-1}) and half lives ($t_{1/2}$) of 17α-and 17β-trenbolone and trendione in agricultural soils D36 and C39 under various temperature and water availability.

Soil	Applied chemical	Matric potential (MPa)	Incubation temperature[b] (°C)	Trenbolone				Trendione		
				k_a (h^{-1}) SE	R^2	$t_{1/2}$ (h) (from k_a)	$t_{1/2}$ (h) Observed	k_b (h^{-1}) SE	R^2	$t_{1/2}$ (h) (from k_a)
D36	17α-trenbolone	−0.03	5	0.012 (0.001)	0.81	59	50	0.0035 (0.0004)	0.74	199
		−0.1	5	0.011 (0.001)	0.81	63	50	0.0031 (0.0003)	0.84	225
		−0.03[a]	22	0.179 (0.007)	0.99	3.8	4	0.0670 (0.0056)	0.75	10
		−0.1	25	0.099 (0.011)	0.91	7	7	0.0240 (0.0022)	0.62	29
		−0.5	25	0.083 (0.010)	0.8	8	10	0.0097 (0.0006)	0.87	71
		−0.5	35	0.130 (0.019)	0.85	5	5	0.0464 (0.0038)	0.66	15
		−1	35	0.028 (0.003)	0.92	25	25	0.0335 (0.0024)	0.65	21
	17β-trenbolone	−0.5	35	0.119 (0.019)	0.78	6	5	0.0626 (0.0035)	0.86	11
		−1	35	0.044 (0.006)	0.79	16	15	0.0342 (0.0026)	0.63	20
	Trendione	−0.03[a,c]	22					0.0280 (0.0008)	0.99	24
		−0.5	35					0.0143 (0.0017)	0.8	48
		−1	35					0.0104 (0.0015)	0.61	67
C39	17α-trenblone	−0.03[a,c]	22	0.077 (0.005)	0.96	9	9	0.0143 (0.0013)	0.52	49
		−0.03	5	0.010 (0.002)	0.75	72	48	0.0052 (0.0002)	0.81	133
		−0.1	5	0.009 (0.001)	0.89	79	48	0.0050 (0.0002)	0.8	138
		−0.1	25	0.063 (0.005)	0.95	11	10	0.0571 (0.0042)		12
		−0.5	25	0.036 (0.004)	0.89	19	15	0.0180 (0.0010)	0.81	38
		−0.5	35	0.062 (0.003)	0.97	11	11	0.0340 (0.0027)	0.61	20
		−1	35	0.028 (0.003)	0.91	24	25	0.0171 (0.0012)	0.74	40
	17β-trenbolone	−0.5	35	0.051 (0.005)	0.91	13	20	0.0323 (0.0022)	0.68	21
		−1	35	0.032 (0.004)	0.8	22	22	0.0233 (0.0015)	0.72	30

[a] Data taken from Khan et al. 2008; average incubation temperatures were ±2 °C.
[b] All other incubation temperatures were controlled (with SD ± 0.5) except when indicated otherwise.
[c] Soils (D30 and C32) had similar textural characteristics and were sampled from the same field but at a different time and soils were not amended with manure.

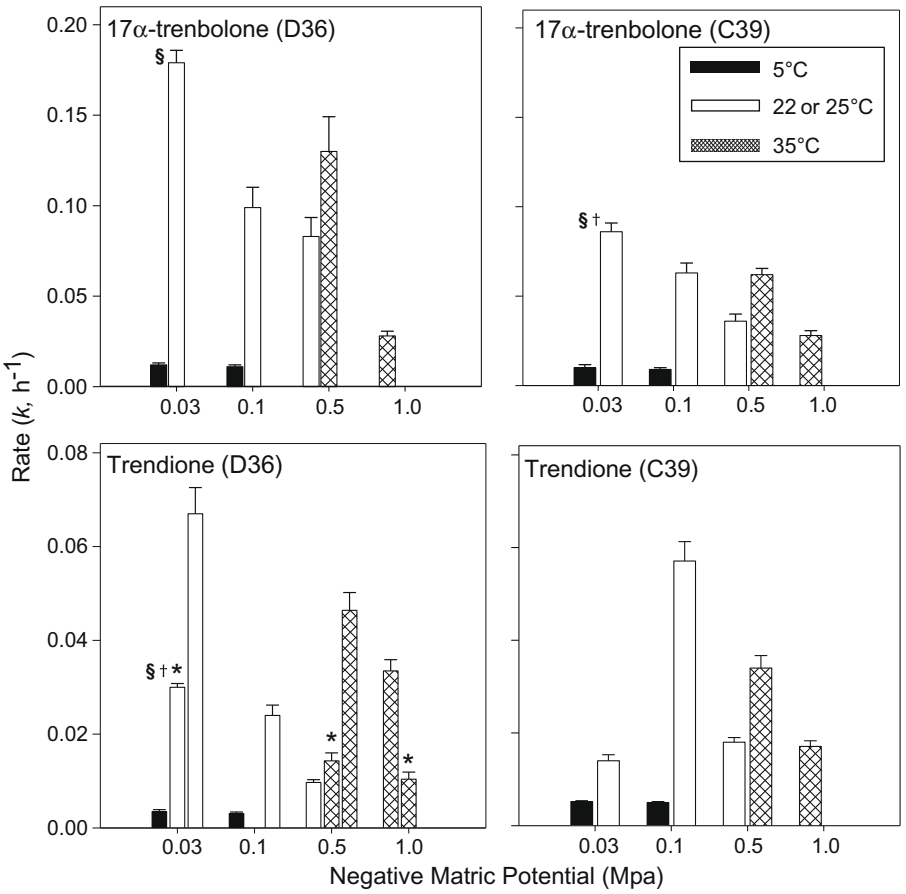

Fig. 1. Effect of soil matric potential and temperature on the degradation rates in soils C39 and D36 of 17α-trenbolone applied to soils at 50 μg kg^{-1} and of the metabolite trendione. Error bars represent the standard errors. *Degradation rates for trendione when applied directly to soils at the same rate (50 μg kg^{-1}); §data from Khan et al. (2008); †soils (D30 and C32) had similar textural characteristics and were sampled from the same field but at a different time and soils were not amended with manure.

soils at a given temperature and moisture; and 17α- versus 17β-trenbolone in a given soil incubated at similar conditions of moisture and temperature. Paired *t*-tests were performed using SAS to determine the significant difference at the 95% confidence level between the paired treatments (Table S1).

3. Results and discussion

Aerobic degradation rates estimated for 17α-trenbolone, 17β-trenbolone and trendione in two agricultural soils under various temperature and matric potential combinations are summarized in Table 3 and Fig. 1. Representative patterns of 17α-trenbolone degradation to trendione with subsequent degradation of trendione are shown in Fig. 2 for both soils incubated at −0.1 MPa and 25 °C.

3.1. Trenbolone degradation

Effect of temperature was assessed at matric potentials of −0.03, −0.1, −0.5 and −1.0 MPa. In all cases, degradation rates of 17α-trenbolone increased with increasing temperature (Fig. 1A and B) with the greatest difference observed at −0.03 MPa. At higher temperature (35 °C) combined with very dry conditions (−1.0 MPa) slowed degradation as previously observed for the natural androgen testosterone in an agricultural soil (Lorenzen et al., 2005). Soil matric potential influences microbial activity by modifying substrate availability hence reducing their activity (Zak et al., 1999; Lee et al., 2004). Temperature dependence can be estimated quantitatively by applying the Arrhenius equation, $k = Ae - \frac{E_a}{RT}$, the frequency factor and E_a is the activation energy (Schwarzenbach et al., 2003). We did not more than two temperatures for a given matric potential; however, in many cases the data at a given temperature were similar for −0.1 and −0.5 MPa allowing the pooling of temperature data collected for 17β-trenbolone at these two matric potentials (see Supporting material, Table S2) to estimated E_a values. The resulting ln k vs $1/T$ plots, where E_a/R is the slope and A is the intercept were linear with goodness-of-fits ranging from 0.72 to 0.96 for 17α-trenbolone and trendione produced from 17α-trenbolone in the two soils (see Supporting material, Fig. S4). For a given soil, the resulting E_a/R values are similar for both compounds with E_a values of ~62 kJ mol^{-1} and ~49 kJ mol^{-1} in D36 and C32, respectively (see Supporting material, Table S3). These E_a values can be used to estimate changes in degradation rates with changes in temperature.

At a given temperature, hormone degradation rate for all three androgens decreased as the soil's matric potential became more negative (decreasing water availability). Degradation rates and trends were similar between trenbolone isomers. Slower degradation was generally observed for all three androgens in the sandy soil (C39) compared to the higher OC silty clay loam (D36) (statistically significant at 95% CI; Table S1) except under the more extreme conditions. No significant difference between soils was observed for any of the androgens for the coldest condition (at 5 °C) or for either trenbolone isomer under the hottest and driest condition (35 °C and −1.0 MPa). Typically sorption often limits

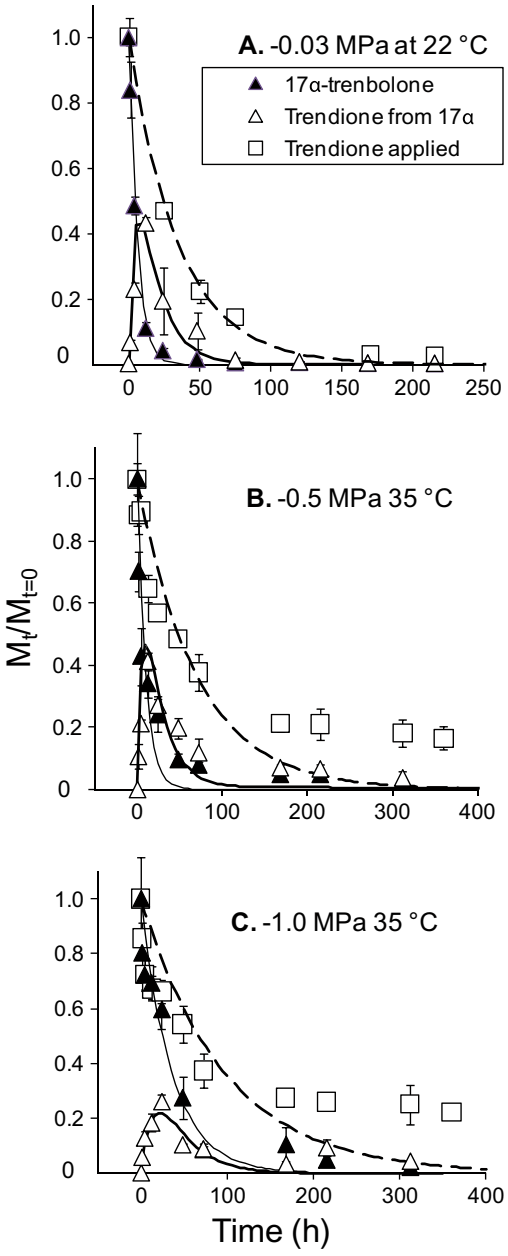

Fig. 2. Moles over time (M_t) relative to applied moles ($M_{t=0}$) for degradation of 17α-trenbolone applied at 50 μg kg^{-1}, trendione from 17α- trenbolone, and trendione applied at 50 μg kg^{-1} to soil D36 incubated at (A) −0.03 MPa and 22 °C; (B) −0.5 MPa and 35 °C; and (C) −1.0 MPa and 35 °C. Error bars represent the standard deviation of triplicate samples.

degradation rates for many organic compounds (Ogram et al. 1985; Weissenfels et al. 1992); however, C39 which sorbs three times less than the higher OC clay loam D36 (Khan et al., 2009), exhibited the slower degradation rates. The faster degradation in D36 may be due to higher microbial numbers or microbial diversity associated with higher OC soils, which were not directly measured in this study.

3.2. Trendione degradation

Trendione exhibited similar dependency on available water content and temperature as the trenbolone isomers; however, trendione consistently persisted longer than trenbolone under all conditions (Fig. 1C and D, Table 2). Trendione is 2–4 times more sorptive than 17α- and 17β-trenbolone, respectively (Khan et al., 2009) with average log OC-normalized distribution coefficients (log K_{oc}, L kg^{-1} OC) of 3.38 ± 0.19 for trendione, 3.08 ± 0.1 for 17β-trenbolone and 2.77 ± 0.12 for 17α-trenbolone. Also when trendione was applied directly to soil, it degraded two to three times slower than when it was degraded after being formed as a degradation product of trenbolone. This study was not explicitly designed to assess the role of sorption on degradation; however, the potential effect of sorption and desorption rates on degradation as it pertains to intracellular versus extracellular enzymatic degradation is discussed in the next section.

3.3. Degradation of applied versus produced trendione

A number of explanations are plausible for why trendione degradation was slower when applied directly to soil compared to when it was produced form trenbolone degradation including concentration effects, availability, and enzyme activation. In soil microcosms where trendione was applied directly, initial soil concentrations were 50 μg kg^{-1} whereas when trendione was produced as a degradation product, the highest concentration present was no more than half this loading. Khan et al. (2008) reported a decrease in trendione degradation with increasing concentrations; however, degradation was only retarded by a factor of four over an applied concentration range of nearly two orders of magnitude (40–3000 μg kg^{-1}).

Conversion of 17-hydroxyl steroids takes place by the action of a dehydrogenase enzyme (Talalay, 1957). Enzymatic reactions start with the attachment of enzyme to a substrate molecule. Microbial enzymes are produced intracellularly by soil microbes and are deliberately released by the cells into their nearby environment (Gianfreda and Rao, 2004); therefore, steroid conversion in soils may occur extracellularly, at the external cell wall interface, and intracellularly if the steroid molecules can diffuse across the cell membrane. Extracellular enzymatic reactions may occur with enzymes present in the aqueous or solid phase in soil or attached to the outer cell membrane (Burns, 1982; Gianfreda and Rao, 2004). Intracellular enzymatic reaction requires substrate molecules to first cross the cell membrane usually via diffusion, thus slow diffusion from soil could limit the rate of degradation. Therefore, if intracellular conversion does dominate trenbolone degradation, then this would explain the faster degradation of trendione when produced form trenbolone. Given how fast trenbolone can degrade (half life in optimal conditions is less than 3 h), diffusion through the cell wall would have to be rapid, which may be highly questionable given its size; however, steroid hormones are lipophilic molecules and have been shown to rapidly cross biomembrane barriers in fungi (Oren et al., 2004; Cresnar and Zakelj-Mavric, 2009). In this case, trendione produced from trenbolone would already be present in the cell where there is a high concentration of enzymes present, thus degrading at a faster rate compared to when it is directly applied to the soil where it would have to first diffuse across the cell wall. Alternatively, degradation at the external cell wall interface could also result in trendione degradation being faster when produced by trenbolone relative to being applied for the same reasons, trendione applied directly would have to first diffuse to the external cell wall interface. Additional degradation in the soil matrix once enzymes have been excreted from the cell may be limited by biodegradation of the enzyme itself.

The potential effect of sorption and desorption rates on degradation is further exemplified in the fits from the first-order degradation model. Model fits to the data generally appear good over the incubation period for both trenbolone and trendione at −0.03 MPa (Fig. 2A). However, at more negative matric potentials, the measured hormone concentrations during the latter part of the incuba-

tion period (e.g., >50 h) are greater than predicted by Eqs. (1) and (2), and the differences are much greater for trendione (Fig. 2B and 2C). This is likely due to the fact that with increasing residence time, hormones can penetrate deeper into the soil matrix, such that sorption may begin to significantly limit degradation (Hatzinger and Alexander, 1995). Also subsequent desorption in soils with low moisture contents will be retarded, because of limited connectivity between water filled pores and soil particles. The model (Eqs. (1) and (2)) do not account for sorption or any mass transfer limitations that may affect degradation. For both intracellular and extracellular degradation, increasing residence time of a chemical within the soil matrix during which a chemical may diffuse deep into soil pores, can lead to a further reduction of substrate bioavailability (Scow and Alexander, 1992; Alexander, 2000). The latter may explain the increasingly larger apparent resistant fraction of trendione at the high matric potentials (low moisture content, thus pore connectivity).

There are other plausible explanations for the difference in observed rates between trendione produced from trenbolone versus applied directly. In many instances the enzymes involved in microbiological steroid transformations are induced (adaptive), and may be raised to high levels when microorganisms are exposed to a steroid substrate. If trenbolone induces enzyme production more rapidly than trendione, then trendione has the potential to degrade more rapidly in the presence of trenbolone. Also an enhancement in secondary substrate metabolism in the presence of an easily metabolized primary substrate (that may also supported microbial growth) has been hypothesized and reported in other studies (Lapat-Polasko et al., 1984). Lastly, it is also plausible that although we assumed no microbial growth during the 10 d of incubation with the applied hormones, there may indeed be some growth. However, given the small amounts of carbon provided by the trenbolone addition relative to available soil and manure carbon, this is unlikely.

3.4. 17α-Trenbolone conversion back to 17β-trenbolone

17α-Trenbolone converted back to 17β-trenbolone (<1.5%) under optimal conditions (−0.03 MPa or −0.1 MPa and ~25 °C similar to what was observed in our earlier work (Khan et al., 2008). In the current work, some conversion was also observed at 5 °C but to a lesser extent (<1% and close to LOD). However, under hot (35 °C) and dry soil (−0.5 and −1.0 MPa) conditions, no conversion was detected. 17β-trenbolone produced may have been below our LOD or there was no conversion. The latter is plausible if 17α- is converted to 17β- via trendione and trendione is hydroxylated in aerobic soil by fungi, since fungal steroid hydroxylation activity slows down significantly at higher temperatures (*i.e.*, 35 °C) (Singh et al., 1968). Other studies have also shown that soil fungal growth rate is more sensitive than bacteria to soil temperatures higher than 30 °C with the ratio of bacterial to fungal growth rates increasing at temperatures above 30 °C (Pietikäinen et al., 2005). Also unlike bacteria, fungi were found to be more adapted to low temperatures (0–5 °C in soil) (Pietikäinen et al., 2005).

3.5. Summary and implications

Although trenbolone and its metabolite degrade relatively fast under optimal soil temperatures and moisture (Khan et al., 2008), both persist longer in soil when conditions are dry and hot or cold. Estimated half life ($t_{1/2}$) increased from a few hours at favorable moisture and temperature conditions to a few days for trenbolone and to more than a week for trendione under colder conditions (5 °C). Degradation rates significantly decreased at the colder temperature (5 °C) even if moisture contents were favorable. Colder soil temperatures (5 °C) decrease microbial physiological activity and substrate demand (e.g., samples are often stored at 4 °C to minimize microbial degradation). Such conditions are likely after fall manure-application, since this application is typically planned after temperatures drop such that nutrients will be preserved until temperatures warm in the spring prior to planting. Fall manure-application is followed by a decrease in temperature.

Colder temperatures may affect sorption, soil bacterial activity (Zak et al. 1999), and enzyme activity (including extracellular enzymes) in soil (Brzezińska et al., 1998). Lower temperatures typically result in higher sorption (Woodburn et al., 1989), which may reduce steroid availability. In our microcosms (5 g soil at field capacity), the hormone fraction in the water phase ($f_{i,w}$) is small (<0.01) regardless of temperature, thus changes in sorption will not significantly affect $f_{i,w}$ (see Supporting Material). Therefore, effect of colder temperatures on bacteria and enzymatic activity likely plays the major role in reducing degradation at lower temperatures. Dehydrogenase activity has been reported to be affected by decreasing soil temperature and moisture content (Trevors 1984). Increase in temperature from 10 °C to 20 °C caused an increase in dehydrogenase activity by a factor of 3.2, whereas combined effect of flooding and temperature increase (30 °C) increased its activity by 129 fold compared to 10 °C and dry conditions (Brzezińska et al., 1998). So under cold conditions, hormone sorption may be enhanced; however, decreases in extracellular enzyme activity and slow bacterial physiological activity are the limiting factors that could potentially lead to increased persistence in soil. Although higher temperature favor faster degradation if soil moisture is not limiting, if both rainfall is low while temperatures are high such as can occur for some periods during the summer, hormone persistence is also likely to increase. For example, 17α-trenbolone persistence when incubated at 35 °C, increased by 2–5 times when matric potential decreased from −0.5 MPa to −1.0 MPa in the sandy soils to silty lay loam, respectively (Table 3).

Conditions that lead to increased persistence will increase the time for hormones to sorb and diffuse into to soil particles, thus potentially reducing hormone leachability. Depending on the timing and intensity of a rainfall event and temperature conditions, hormones may desorb upon a rainfall event and subsequently be degraded or mobilized. Compared to trenbolone, trendione, which degrades more slowly and appeared to have a significant fraction resistant to degradation in the laboratory-based microcosms, mobilization to nearby water bodies may be more likely. Although trendione is reported to be less potent than 17β-trenbolone based it's on binding affinity to mammalian androgen receptor (Bauer et al., 2000), its potential to cause reproductive effects in aquatic organisms should not be ignored. These metabolites could be equipotent as their parent hormones to aquatic species as observed for estrone, a metabolite of estradiol (Metcalfe et al., 2001). Although estrone was found to be less estrogenic than 17β-estradiol using a YES assay, the lowest observed effect levels (LOEL) for induction of ova in testis of male Japanese medaka was same (0.01 μg L^{-1}) for both estrone and 17β-estradiol (Metcalfe et al., 2001).

This study focused only on the conditions typical of the upper soil horizons and did not include saturated conditions such as may occur after a heavy rainfall (partially flooded field) or as exist for sediments in streams. Sediments in streams become anaerobic within a few millimeters from the sediment surface (Kunkel and Radke, 2008), which is not favorable for hormone degradation and mineralization, thus allowing hormones to persist longer (Schiffer et al., 2001; Ying et al., 2004; Hakk et al., 2005). In addition, anaerobic conditions favor conversion of oxidized hormone metabolites such as trendione and estrone back to the parent hormone. Such conversion was observed for estrogens; Joss et al. (2004) observed 17β-estradiol produced when in an estrone spiked sewage sludge (under anaerobic conditions). Czajka and Londry (2006) observed the conversion of estrone to estradiol in a lake

water and sediment culture under reducing conditions. If conversion back to the more active parent hormones occurs within stream sediments, these sediments may serve as a long term source to the water column. Information is lacking on the behavior of synthetic and natural hormones and their metabolites in anaerobic sediments.

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Appendix A. Supplementary material

Information on trendione synthesis, effect of hormone carried, extraction efficiency, LC/MS matrix effects, comparative statistics, Arrhenius relationship, and effect of sorption can be found online. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chemosphere.2010.02.036.

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